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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Silver Nanoparticle–Induced hMSC Proliferation Is Associated with HIF-1α-Mediated Upregulation of IL-8 Expression

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TO THE EDITOR

A recent study has reported that the application of silver nanoparticles (AgNPs) enhances wound healing (Tian *et al.*, 2007). Human mesenchymal stem cells (hMSCs) could enhance normal and impaired wound healing (Shin and Peterson, 2013). A previous paper showed that AgNPs might exert cytotoxic effects on hMSCs at high concentrations, but also induce cell activation at subtoxic silver concentrations (Greulich *et al.*, 2009; Hackenberg *et al.*, 2011). Nonetheless, the effects of AgNP-treated hMSCs in wound healing remain unknown.

Rennekampff *et al.* (2000) reported that IL-8 might be a growth factor for the epithelium. It has been suggested that AgNPs could contribute to wound healing by increasing cell proliferation via upregulation of IL-8 (Hackenberg *et al.*, 2011). However, there is a lack of information on the regulatory mechanism of IL-8 in AgNP-treated hMSCs.

Hypoxia-inducible factor-1α (HIF-1α) acts as transcription factor in regulating metabolism, development, proliferation, and pathology under hypoxic conditions (Liu *et al.*, 2013). Yu *et al.* (2007) also demonstrated that wound healing in diabetic patients is associated with an increase in the synthesis of HIF-1α protein. In this study, we investigated the role of HIF-1α in AgNP-mediated cell proliferation and IL-8 upregulation in hMSCs.

The present study measured cell proliferation and the expression of HIF-1α and IL-8 in hMSCs treated with AgNPs. In addition, this study investigated the effect of small interfering RNA specific for HIF-1α (HIF-1α siRNA) on cell proliferation and IL-8 expression in hMSCs. Polyvinylpyrrolidone-coated spherical AgNPs (nanoComposix, San Diego, CA) were used in this study. AgNPs were characterized by transmission electron microscopy before performing the experiment (Figure 1a). The energy diverse X-ray spectra demonstrated the strong presence of Ag (Figure 1b). In accordance with a previous study (Hackenberg *et al.*, 2011), AgNPs increased cell proliferation in a dose-dependent manner at subtoxic concentrations and decreased cell proliferation at higher concentrations above $10\,\mu g\,ml^{-1}$ (Figure 1c and e). As shown in Figure 1d and f, a marked increase in cell proliferation was observed after treatment with AgNPs. To determine the effect of AgNPs on IL-8 mRNA expression, we treated hMSCs with

Abbreviations: AgNP, silver nanoparticle; hMSC, human mesenchymal stem cell; HIF-1α, hypoxia-inducible factor-1α

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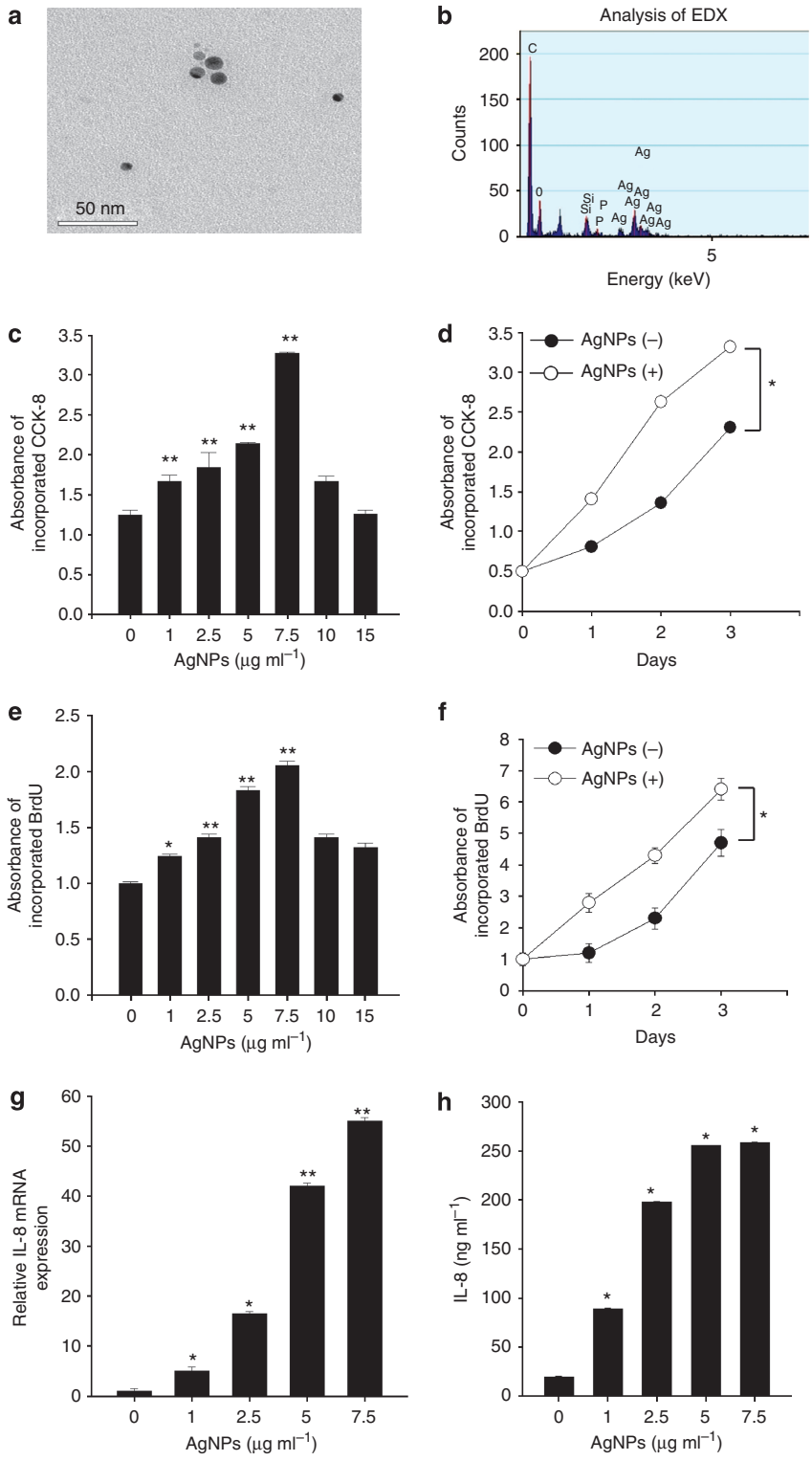

Figure 1. Silver nanoparticles (AgNPs) increase the proliferation of human mesenchymal stem cells (hMSCs) and the gene expression and production of IL-8 in hMSCs. (**a**) Transmission electron microscopy images revealed that AgNPs had a spherical shape and measured 9.5 ± 1.7 nm in diameter. (**b**) The energy diverse X-ray (EDX) spectrum for the particle revealed the strong presence of silver (Ag). hMSCs were treated with various concentrations of AgNPs for 48 hours, and cell proliferation was measured by CCK-8 (**c**) and BrdU (**e**) assays. Cells were also treated with 5 μg ml^{-1} AgNPs for designated times, and cell proliferation was measured by CCK-8 (**d**) and BrdU incorporation (**f**). (**g**) hMSCs were treated with various concentrations of AgNPs for 48 hours. Real-time PCR was performed to analyze the changes in IL-8 gene expression. (**h**) hMSCs were treated with various concentrations of AgNPs for 48 hours and the amount of IL-8 released into the culture supernatants was determined by ELISA. *P<0.05, **P<0.01. Each bar represents the mean ± SD of three independent experiments.

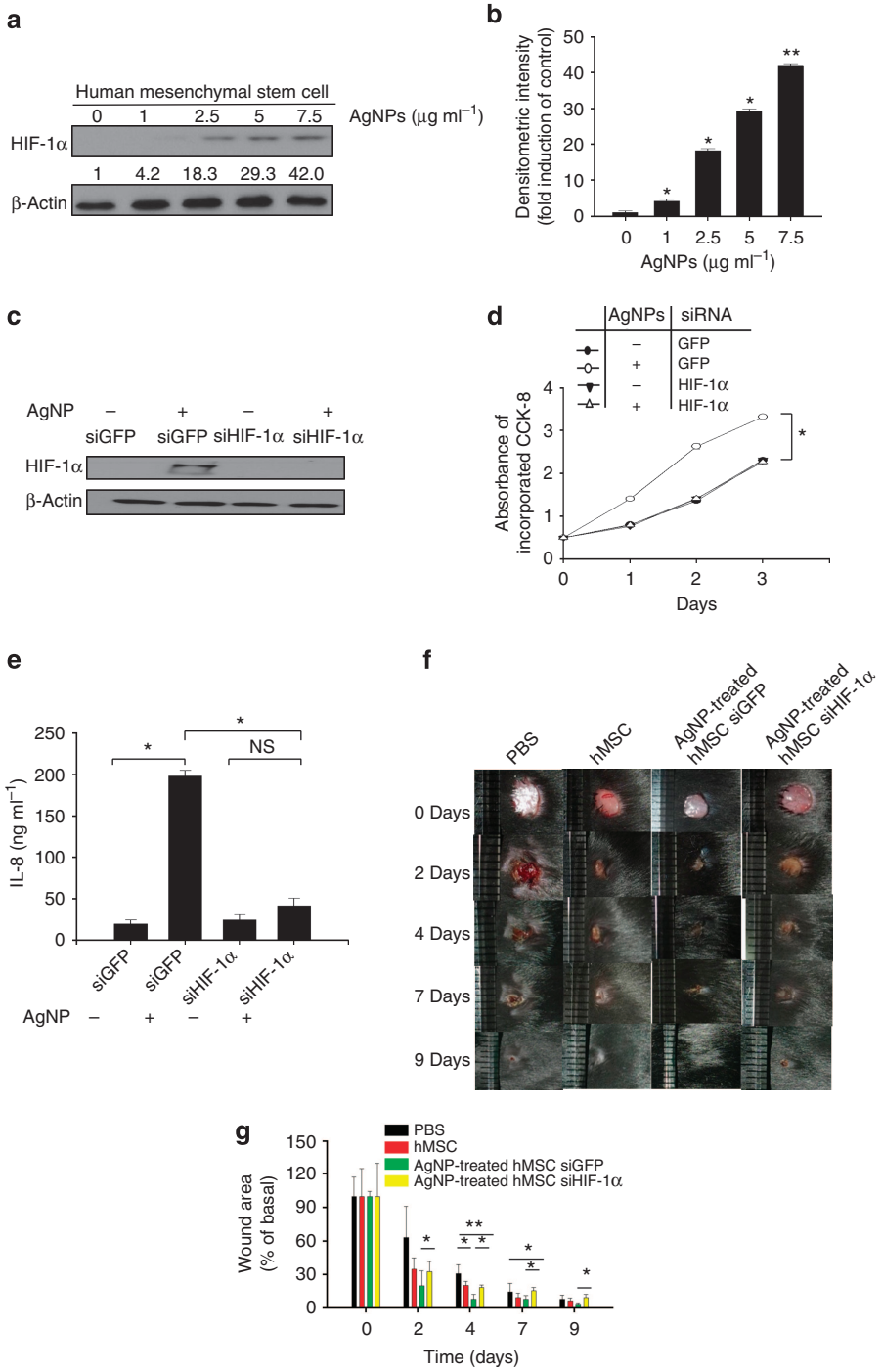

Figure 2. Knockdown of hypoxia-inducible factor-1α (HIF-1α) expression can attenuate the beneficial effects on skin wound healing mediated by silver nanoparticle (AgNP)-treated hMSCs. (**a**) Human mesenchymal stem cells (hMSCs) were treated in the presence of various concentrations of AgNPs for 48 hours, and the expression of HIF-1α in cell lysates was determined by western blot. (**b**) AgNPs induced the expression of HIF-1α protein in a dose-dependent manner. (**c**) The expression of HIF-1α protein in lysates of hMSCs transfected with HIF-1α or control small interfering RNA (siRNA) constructs was measured by western blotting. siRNA-treated hMSCs were treated with AgNPs for 48 hours. β-Actin was used as a loading control. (**d**) AgNP-mediated hMSCs proliferation was diminished in hMSCs treated with HIF-1α siRNA. (**e**) hMSCs treated with HIF-1α siRNA were incubated in the presence of AgNPs for 48 hours, and the concentration of IL-8 released into the culture supernatants was determined by ELISA. AgNP-induced IL-8 expression was reduced in hMSCs treated with HIF-1α siRNA. (**f**) An E-PL3 digital camera (Olympus, Tokyo, Japan) was used to record images of wounds on days 0, 2, 4, 7, and 9. (**g**) The wound-healing rates were evaluated using image analysis. Wound healing in AgNP-treated hMSCs with control siRNA mice was significantly increased on days 2, 4, 7, and 9, compared with AgNP-treated hMSCs with HIF-1α siRNA ($n = 6$). *$P < 0.05$, **$P < 0.01$, NS, not significant, $P > 0.05$. Each bar shows the mean ± SD from three separate experiments. GFP, green fluorescence protein; PBS, phosphate-buffered saline.

AgNPs for 24 hours and performed real-time reverse transcription-PCR to evaluate mRNA expression of IL-8. IL-8 mRNA expression was significantly increased in the hMSCs treated with AgNPs (1, 2.5, 5, and 7.5 μg) compared with the untreated hMSCs ($P<0.05$ for all comparisons; Figure 1g). ELISA analysis also showed that AgNP-induced IL-8 secretion from hMSCs in a dose-dependent manner (Figure 1h).

After exposure to AgNPs, the expression of HIF-1α protein in hMSCs increased in a dose-dependent manner (Figure 2a and b). To investigate the role of HIF-1α in AgNP-mediated upregulation of IL-8 and cell proliferation in hMSCs, we treated the cells additionally with HIF-1α siRNA. As shown in Figure 2c, expression of HIF-1α protein in AgNP-treated hMSCs was significantly reduced after HIF-1α siRNA treatment, confirming that HIF-1α siRNA effectively blocked the expression of HIF-1α in these cells. The results showed that AgNP-mediated hMSC proliferation was decreased in hMSCs treated with HIF-1α siRNA (Figure 2d). We measured AgNP-induced IL-8 expression in hMSCs that were transfected with HIF-1α siRNA by ELISA and demonstrated that IL-8 production was also reduced following treatment with HIF-1α siRNA (Figure 2e). Six-week-old female C57BL/6 mice (Orientbio, Sungnam, Korea) were maintained and handled under protocols approved by the Korea University Institutional Animal Care and Use Committee (KUIACUC-2013-243). These mice averaged 18 g. The mice were divided into four different groups, with six animals in each group. Wounds were made on the dorsal skin of each mouse using a 5 mm punch. According to their respective groups, the wound site was injected with phosphate-buffered saline, hMSCs, AgNP-treated hMSCs with HIF-1α siRNA and AgNP-treated hMSCs with control siRNA. Our preliminary studies have shown that immunosuppressive therapies were not needed in hMSCs treatment. The wound areas were evaluated using image processing techniques of Matlab (The MathWorks, Natick, MA). This study showed that AgNP-treated hMSCs could accelerate cutaneous wound healing *in vivo*, compared with untreated hMSCs (Figure 2f and g). In addition, our findings presented delayed wound closure in the AgNP-treated hMSCs with HIF-1α siRNA group compared with the AgNP-treated hMSCs with control siRNA group (Figure 2f and g). The present results suggest that accelerated wound closure by injecting with AgNP-treated hMSCs may be HIF-1α dependent. Histopathologic examination demonstrated that hair growth and scar formation might be major factors for the effects of AgNP-treated hMSCs in wound healing (Supplementary Figure S1 online).

One study showed that hypoxic conditions induce extracellular matrix remodeling via HIF-1 in equine dermal fibroblasts (Deschene *et al.*, 2012). Du *et al.* (2013) demonstrated that HIF-1α is important for burn wound healing in a mouse model through HIF-1α gene therapy. Rezvani *et al.* (2011) showed that HIF-1α could enhance the wound-healing process in several ways, including vascular endothelial growth factor release and triggering the mobilization of angiogenic cells.

Chang *et al.* (2013) demonstrated that HIF-1α is important for MSC survival under hypoxic conditions. In addition, Razban *et al.* (2012) showed that HIF-1α overexpression induced angiogenesis pathways under hypoxic conditions in MSCs. Forristal *et al.* (2013) demonstrated that HIF-1α protein stabilization increases hematopoietic stem cell quiescence and accelerates blood recovery after severe irradiation *in vivo*. In addition, a previous study showed that various aspects of stem cell biology, such as differentiation and mobilization, involve the HIF-1 signaling pathway (Liu *et al.*, 2011). Lim *et al.* (2012) demonstrated that AgNPs induced HIF-1α activation in *Caenorhabditis elegans*. However, to our knowledge, this is the first study to elicit that AgNPs might induce the expression of HIF-1α protein in hMSCs.

The present study suggests that HIF-1α may be a key factor in AgNP-induced cell proliferation and IL-8 expression in hMSCs. In view of these findings, HIF-1α and the related downstream molecules might be an important target of research and development to improve wound healing by using AgNP-treated hMSCs.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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